The Receptor Guanylyl Cyclase C: Insights into Expression and Regulation

Cells are under constant exposure to the external environment and have the ability to respond to changes through a complex process termed as "cellular signal transduction" Control of this life sustaining activity is mediated by the concerted actions of hormones and neurotransmitters Cyclic GMP (cGMP), a ubiquitous second messenger, has an important role to play in cellular signal transduction. Cyclic GMP is synthesized by both soluble guanylyl cyclases and receptor guanylyl cyclases Receptor guanylyl cyclases have a modular domain organization, and are divided into an extracellular ligand-binding domain, a single transmembrane domain, a region with sequence similarity to that of protein kinases, the kinase homology domain (KHD) and a C-terminal guanylyl cyclase domain. The independent structural domains in receptor guanylyl cyclases do not function as completely separate entities, but perform interdependent functions to ensure precise modulation of the catalytic activity.

Guanylyl cyclase C (GC-C) is the membrane-bound receptor for the heat-stable enterotoxin (ST) peptides and for the endogenous peptide ligands guanylin, uroguanylin, and lymphoguanylin. GC-C is predominantly expressed on the apical surface of epithelial cells of the intestine, and activation of GC-C by ST leads to an increase in intracellular cGMP levels Cyclic GMP cross-activates protein kinase A and protein kinase G, which phosphorylate and activate the cystic fibrosis transmembrane conductance regulator (CFTR) leading to chloride ion secretion. This in turn causes secretion of water into the lumen, which results in "traveller's diarrhoea" associated with the ST peptides. The endogenous peptides guanylin and uroguanylin have a lower affinity for GC-C than the ST peptides and are produced within the intestinal mucosa to serve as paracrine and autocrine regulators of intestinal fluid and electrolyte secretion.

Adenine nucleotides have been shown to regulate the activity of GC-C, presumably by binding to the KHD. Structural modelling studies have suggested that the KHD of GC-C can adopt a structure similar to that of protein kinases, and that Lys516, corresponding to the conserved lysine in the protein kinases, is essential for mediating ATP effects in GC-C. In the current study the role of the KHD in GC-C signalling has been investigated. Earlier studies have shown contrasting results that deletion of the KHD in GC-C either results in a constitutively active or an inactive receptor To further delineate the role of this domain, a deletion (491-568 amino acid residues of GC-C)

containing the potential ATP binding site (Lys516) was generated This deletion in the KHD resulted in significant inactivation of GC-C, both in terms of ligand-stimulated guanylyl cyclase activity and activity in the presence of MnGTP as substrate.

Although the KHD has sequence similarity to the catalytic domain of protein kinases, it lacks the critical histidine-arginine-aspartate (HRD) motif that has been shown to be essential for phosphor-transfer activity in protein kinases. This is replaced by histidine-glycine-arginine (HGR) in GC-C. Given the apparent structural homology between the KHD of GC-C and protein kinases, it would be interesting to examine the possibility of introducing the critical HRD motif in GC-C and study how that would modulate the activity of GC-C. Through site-directed mutagenesis, Gly613 and Arg614 residues in GC-C were mutated to Arg and Asp respectively. Interestingly, this mutation also resulted in significant inactivation of GC-C. When the invariant kinase residues are either mutated or introduced in the KHD, there is a loss of ligand stimulated activity, suggesting that any perturbation in sequence results in loss of signal transmission from the extracellular ligand binding domain to the intracellular domain. This could suggest that the KHD of GC-C has evolved to function as a regulatory domain rather than a typical kinase, and may provide an explanation for the presence of a pseudokinase domain in a number of signalling proteins.

In typical kinases, "open" or "closed" structures are observed in the absence or presence of ATP respectively, and this conformational flexibility is a central feature of the regulatory mechanism. Earlier studies have shown that ATP could interact with the KHD and modulate GC-C activity by altering the oligomeric state, which could be associated with a dramatic alteration in the conformation of the receptor. Interestingly, a monoclonal antibody, GCC:4D7, raised against the KHD of human GC-C (460 to 784 amino acid residues) and available in the laboratory, does not bind to GC-C in the presence of ATP. In addition, mutant GC-C_{K516A} failed to react with the GCC:4D7 monoclonal antibody. The loss of reactivity could be either due to inaccessibility of the epitope because of conformational changes brought about upon ATP binding or due to direct ATP binding to the epitope. Epitope analysis by phage display revealed that 517DLKHND522 is the epitope of GCC:4D7, which is present immediately C-terminal to Lys516. The loss of reactivity of the antibody in the presence of ATP was further found to be an inherent property of the paratope of GCC:4D7, since both the whole antibody and the derived ScFv failed to recognize GC-C in the presence of ATP. These findings indirectly indicate

that on binding of ATP to the KHD, there could be conformational changes as observed in typical kinases, which alter the reactivity of the antibody, and these conformational changes in turn regulate receptor activity.

Since the KHD has a regulatory role, compounds that interact with the KHD could serve as regulators of GC-C activity. To investigate this, it was decided to study the effects of different protein kinase inhibitors that either act as ATP analogues or substrate mimics, on GC-C activity. Interestingly, Tyrphostins, potent tyrosine kinase inhibitors, were found to inhibit GC-C activity unlike other kinase inhibitors such as staurosporine and genistein. Tyrphostin A25 inhibited the guanylyl cyclase activity of GC-C *in vitro*, and no inhibition was seen with an inactive analogue, Tyrphostin A1, indicating the requirement of a specific side chain in the Tyrphostins to bring about inhibition. Inhibition was reversible and non-competitive with respect to the substrate (MnGTP).

The KHD could be the site for Tyrphostin binding, thereby modulating cyclase activity. To assess this, the guanylyl cyclase domain of GC-C was expressed in insect cells and was found to be catalytically active. Tyrphostin A25 inhibited the catalytic activity, indicating that the cyclase domain of the receptor was the site for Tyrphostin binding. In agreement with this, the cyclase activities of membrane-bound guanylyl cyclase A and soluble guanylyl cyclase were also inhibited.

In order to evaluate the structural determinants of the basic Tyrphostin skeleton that are required for the inhibition of guanylyl cyclase activity, the effects of a variety of Tyrphostins in bringing about the inhibition of guanylyl cyclase activity were investigated. Interestingly besides Tyrphostin A25, related compounds such as AG18, AG82, AG213, AG490, AG494, AG555, AG556 and AG1288 also inhibited GC activity, compared to others (AG9, AG43, AG370 and AG879) which inhibited the activity poorly. These findings indicate that the hydroxyl groups present in active Tyrphostins probably act by forming critical hydrogen bonds at the Tyrphostin binding site in GC-C, and appear to be essential for inhibition.

Tyrphostin A25 did not inhibit ST-mediated activation of GC-C when applied to intact cells. Interestingly, Tyrphostin AG555 inhibited ST-mediated activation of GC-C in intact cells, and the inhibition was reversible. Tyrphostin AG555 did not have any effect on soluble guanylyl cyclase activity. These results caution against the interpretation of cellular experiments using Tyrphostins as being solely due to inhibition of tyrosine kinases. Effective inhibitors of specific guanylyl cyclase signalling pathways could

provide information in determining the functions of cGMP and of the individual cyclase receptors in many cells throughout the body where the roles of this cyclic nucleotide are not understood. Therefore novel inhibitors of guanylyl cyclases can be synthesized using the Tyrphostins as a backbone.

The major site of expression of GC-C is the intestinal epithelial cell, although GC-C is also expressed in extraintestinal tissue such as the kidney, airway epithelium, perinatal liver, stomach, brain, and adrenal glands. The function of GC-C in these tissues is not clearly understood. So as to investigate the existence of GC-C signalling pathway and its possible role in extraintestinal tissues, it was decided to study the presence of GC-C, uroguanylin, guanylin mRNA, and protein kinase G-II in both male and female reproductive organs and results indicated expression of the mRNA for all these genes in the reproductive tissue of the male and female rat. Based on the observation that CFTR plays an important role in male reproduction and the epididymis is highly dependent on CFTR for its function, it is possible to suggest that the GC-C signalling pathway may also regulate fluid and electrolyte balance of epididymis in an endocrine/paracrine fashion.

Western blot analysis using a monoclonal antibody to GC-C revealed the presence of differentially glycosylated forms of GC-C in the caput and cauda epididymis. Exogenous addition of uroguanylin to epididymal minces resulted in cGMP accumulation, suggesting an autocrine or endocrine activation of GC-C in this tissue. Immunohistochemical analyses using the monoclonal antibody GCC:4D7 demonstrated expression of GC-C in the tubular epithelial cells of both the caput and cauda epididymis. Functional GC-C was also expressed in the immature rat epididymis and was localised in both tubular epithelium and the smooth muscle lining of the tubule.

In intestinal cells, the GC-C promoter is regulated by hepatocyte nuclear factor 4 (HNF-4) and Cdx2 transcriptional factors. So as to investigate whether GC-C is regulated similarly in the epididymis, primary epithelial cultures from the mature rat epididymis were established. Immunocytochemistry studies using a monoclonal antibody raised against cytokeratin, an epithelial cell marker, revealed the presence of epithelial cells in culture. The presence of GC-C was demonstrated by RT-PCR and immunocytochemistry studies. The presence of HNF-4 and Cdx2 transcriptional factors in this tissue was validated by RT-PCR analysis followed by sequence analysis of the PCR products.

It was of interest to investigate whether HNF-4 and Cdx2 are essential for transcriptional regulation of GC-C in the epididymis. In order to address this, wild-type

GC-C promoter-luciferase constructs along with individual mutations in the HNF-4 and Cdx2 sites were transiently transfected into primary epithelial cells. The promoter which lacked either HNF-4 or Cdx2 binding sites was inactive. This suggests that as in the intestine, transcription of GC-C in epididymis also requires HNF-4 and Cdx2 transcription factors. These findings therefore suggest that the GC-C signalling pathway could converge on CFTR in the epididymis and perhaps control fluid and ion balance for optimal sperm maturation and storage in this tissue.

In conclusion, the current study has attempted to understand the role of the KHD in GC-C signalling and mechanism of regulation of GC-C activity by ATP. Studies suggest that novel nucleotide cyclase inhibitors based on the Tyrphostin scaffold can be developed. These could aid in a greater understanding of nucleotide cyclase structure and function. The existence of the GC-C signalling pathway in reproductive tissues and in epididymis in particular suggests a novel role for GC-C in controlling germ cell function and maturation.